THE EFFECT OF RESTORING GDNF SIGNALING ON SPERMATOGONIAL STEM CELL DIFFERENTIATION

by
Andrew J. Laychur

A thesis submitted to the Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

July, 2016

ABSTRACT

Spermatogonia Stem cells (SSCs) are the foundation of male fertility. A group of infertile men lack enough SSCs to maintain sperm production, a phenotype labeled Stertoli Cell-Only. Decades of research has implied glial cell line-derived neurotrophic factor (GDNF) as a key factor for sustaining SSC and their immediate progeny undifferentiated spermatogonia, but more work is necessary to determine GDNF's clinical relevance.

This thesis examines the impact of inhibition and then restoration of GDNF on the differentiation of SSC and undifferentiated spermatogonia. Specifically testing the hypothesis that restoring GDNF signaling after 9 days of its inhibition would cause the remaining undifferentiated spermatogonia to suppress differentiation thereby restoring numbers of SSCs and progenitor spermatogonia in the testis. The Wright lab has developed a chemical-genetic approach to inhibit GDNF signaling through its intracellular tyrosine receptor kinase RET. Under normal conditions GDNF signaling proceeds unaffected but in the presence of a bulky ATP competitive-inhibitor signaling is inhibited. This thesis utilized that chemical genetic approach to block GDNF signaling for nine days and collects samples at various time points after GDNF inhibition. The samples were then processed through immunocytochemistry and analyzed using microscopy.

The results show a varied response in differentiation by cell type. One population of undifferentiated spermatogonia, containing mostly progenitor transit amplifying undifferentiated spermatogonia, quickly and drastically decreases differentiation. While

another population, partially comprised of SSC, had little decrease in differentiation with GDNF signal restoration. The combined work implies a restoration of GDNF signaling has different effects on SSC and undifferentiated transit amplifying progenitor spermatogonia. This nuanced response indicates GDNF may act in coordination with other extrinsic signaling molecules in order to sequentially rebuild the numbers of transit amplifying progenitor spermatogonia and then the pool of SSCs.

THESIS ADVISOR: *William Wright, Ph.D.*

SECONDARY READER: *Barry Zirkin, Ph.D.*

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The process of making sperm begins with spermatogonial stem cells (SSCs); these cells are the foundation for male fertility. Normal human males produce as many as 1,000 sperm for every heartbeat from puberty until death. Producing this large number of highly differentiated cells requires a robust and tightly regulated population of stem and progenitor cells (Griswold, Oatley 2013). Our research is concerned with the regulation of SSCs, clinically relevant to a subset of infertile men referred to as Sertoli Cell-Only (SCO) (Del Castillo et al. 1947; Gul et al. 2013). Despite this name, many among these men possess some SSCs, providing hope for treatment (Sibler et al. 2000). Our research strives to elucidate causes and future treatments for these currently incurable cases of male infertility.

Adult stem cells throughout the body are relied upon to replenish tissues with cells that are lost either through terminal differentiation (e.g. formation of sperm) or through injury. Stem cells are capable of dividing symmetrically to produce two new daughter stem cells, thereby increasing stem cell numbers; or asymmetrically, producing one stem cell and one differentiating cell (see Figure 1)(Oatley, Brinster 2012). This maintains stem cell numbers while providing cells that go on to differentiate. In the testis, properly regulated SSCs are the basis for male fertility. However, the study of SSCs is challenging because of the difficulty in distinguishing these cells from their immediate progeny, the transit amplifying progenitor spermatogonia. The classical experimental proof for the existence of stem cells is that upon transplantation, the cells can reconstitute an entire cell lineage. Self-renewing replication of stem cells maintains the stem cell pool, and provides cells that differentiate and generate transit amplifying progenitor

spermatogonia. The transplantation assay involves depleting the testes of naïve spermatogonia and then injecting donor cells into the seminiferous tubules of the testes. Expansion of the injected cells and establishment of spermatogenesis proves the presence of SSCs in the donor sample (Oatley, Brinster 2008; Brinster, Avarbock 1994).

Functioning of stem cells is regulated by extracellular and intracellular signals (Li and Xie, 2005; Underhill and Bhatia, 2007). The stem cell "niche" refers to a defined anatomic region that regulates stem cell renewal and differentiation, and thus how particular stem cells function in tissue maintenance and repair without becoming depleted (Li, Xie, 2005; David, 2006). If there is failure of transplantation assays of normal SSCs into SCO testes (i.e. failure of the injected cells to reestablish spermatogenesis), this implies either that the cells comprising the microenvironment or niche in the testis is defective and thus is responsible for infertility. This makes defining the SSC niche and factors that mediate it critically important for addressing male infertility.

It is clear that the somatic cells of the testicular seminiferous tubules, the Sertoli cells, constitute an essential part of the spermatogonial stem cell niche. Specifically, Sertoli cell products, including glial cell line-derived neurotrophic factor (GDNF) plays an essential role in maintaining SSC number in the adult testis. Our experiment manipulates GDNF, a key factor in the niche, using a novel chemical-genetic approach. This approach allows for pharmacological manipulation of GDNF, and reduction of SSC numbers in a highly controlled manner. Once treatment ends, SSC numbers are rebuilt intrinsically. The goal is to identify molecules and mechanisms responsible for rebuilding a depleted stem cell pool. With this information, we predict that we can then decipher why stem cell numbers do not expand in men with SCO testes. Specifically, the major

experiment in this thesis measures the effects of depletion and then the restoration of GDNF signaling on the differentiation of SSCs and their immediate progeny, the undifferentiated transit amplifying progenitor spermatogonia. We hypothesize that when GDNF signaling is restored after 9 days of inhibition, numbers of SSCs and progenitor spermatogonia are rebuilt through suppressing the differentiation these cells.

BACKGROUND

Spermatogonial stem cells (SSCs) reside in a specific environment of the seminiferous tubules of the testes called the SSC niche. The niche is a physical location in the seminiferous tubules that provides the proper nutrients and signals from the surrounding cells and interstitial environment to maintain self-renewing SSCs (see Figure 2). Sertoli cells most directly influence the niche by providing the physical location in which SSCs are found, by adhering to spermatogonia, and by secreting factors necessary for maintaining their undifferentiated state. These large cells line the seminiferous tubules. Tight junctions form between adjacent Sertoli cells, creating what is referred to as the blood- testes barrier (see Figure 2). The tight junctions create the two compartments within the seminiferous tubules. The area between the basement membrane and the tight junctions formed by Sertoli cells is called the basal compartment of the seminiferous tubules. The basal compartment represents the physical location of the SSC niche. It is separate from the luminal compartment of the tubules where the differentiating cells are undergoing meiosis and forming mature spermatozoa. (Oatley Brinster, 2013). SSCs and progenitor spermatogonia in mouse testes express integrins (α and β), which are known to bind laminins expressed by Sertoli cells (Shinohara et al, 1999). Impaired expression of β1-integrin on SSCs *in vitro* resulted in reduced attachment to laminin molecules and

dramatically reduced the re-colonization of SSCs in recipient testes (Kanatsu-Shinohara et al, 2008). *In vitro* cultures and *in vivo* mouse models have led the field to identify three promising growth factors that maintain SSCs: FGF2, CSF-1, and GDNF. There is compelling evidence for GDNF supporting SSCs, and GDNF's effects on SSC self-renewal are discussed below. FGF2 in addition to GDNF has been shown to be a necessary factor for long-term cultures of SSCs from mouse of specific genetic backgrounds (Kanatsu-Shinohara et al 2005). Colony Stimulating Factor-1 (CSF-1) has been shown to have effects on SSC self renewal in vitro; long-term cultures with CSF-1 yielded an increase in the fraction of SSC verified by transplantation assays (Oatley et al. 2009). Interestingly the total number of undifferentiated spermatogonia was not increased implying CSF-1 specifically affects spermatogonia self-renew and the balance between SSC and transit amplifying progenitor spermatogonia.

Figure 1.

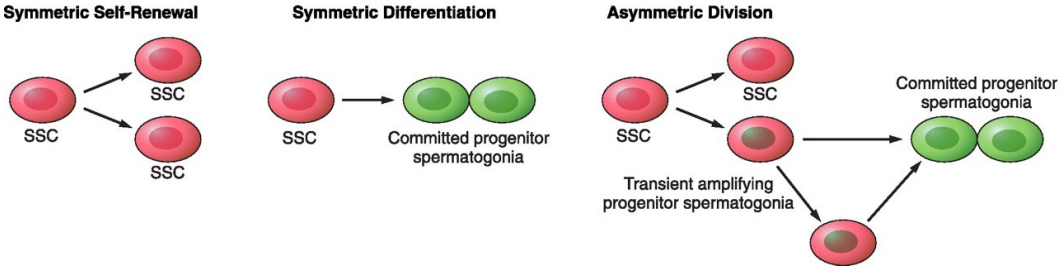


Figure 1. (Oatley, Brinster 2012). *These schematics represent the types of symmetric and asymmetric divisions SSC undergo to self-renew stem cells and maintain spermatogenesis.*

Figure 2.

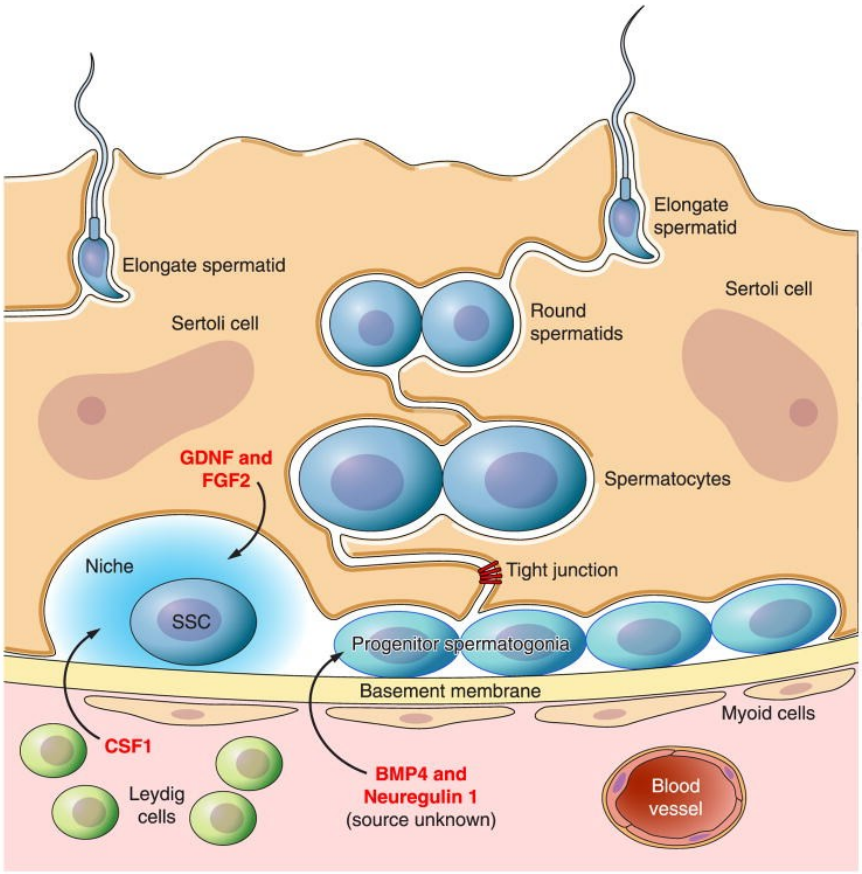


Figure 2. (Oatley, Brinster 2012). *A. This cartoon represents the seminiferous tubule architecture, and the SSC niche with surrounding cells/intersitum that directly or indirectly influence the niche. Stertoli cells adhere to SSC and secrete GDNF, FGF2, among many other factors nurturing SSC to sperm. Leydig cells are known for producing androgens which directly and indirectly support spermatogenesis. Interestingly they also secret CSF-1. The tight junctions between stertoli cells separate spermatogonia from differentiating spermatocytes undergoing meiotic divisions and further differentiation.*

In mice and men, SSCs and progenitor spermatogonia combine to form a population of undifferentiated spermatogonia with specific morphological characteristics. In mice the undifferentiated A spermatogonia have three subdivisions: A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}). The classic model proposes the A_s population contains the SSCs. When these cells divide, they form either more A_s spermatogonia that continue to act as SSCs or they form A_s cells that function as transit amplifying progenitor spermatogonia.

The division of these progenitors forms two linked A_{pr} cells, which then undergo another division forming a chain of A_{al} cells (de Rooij, 2000). These A_{al} can continue to divide in aligned linked chains acting as progenitor undifferentiated transit amplifying cells (Oakberg, 1971). It is important to note that not all A_s cells are SSCs. SSCs as defined by serial transplantation assays number approximately 3,000 in a mouse testis (Nagano, 2003), while the A_s number is around 30,000 per testes (Parker et al. 2014).

Accurately identifying the true SSCs has been challenging because of the difficulty teasing them away from the undifferentiated transit amplifying spermatgonia. A number of potential molecular markers for these cells have been considered, including as GFRα1, PLZF, Neuro3, Nanos2, Lin28, and most recently ID4. Yet none of these has successfully distinguished the SSCs from the undifferentiated progenitor spermatogonia (de Rooji, Griswold 2000). GFRα1 is the cell membrane ligand for GDNF and is commonly used to define a population of undifferentiated SSCs in the testis. GFRα1 is considered a marker of more primitive type A undifferentiated spermatogonia possessing stem cell potential (Buageaw et al, 2005). GDNF's critical role on SSC self-renewal has been elucidated through *in vitro* and *in vivo* experiments. *In vitro* adding GDNF to culture media is necessary for the long-term maintenance and expansion of SSCs (Kubota et al, 2004). *In vivo*, seminiferous tubules of mice overexpressing GDNF had an overproduction of undifferentiated spermatogonia and lacked later stages (Meng et al. 2000). Our lab has shown that inhibition of GDNF signaling for 7 days leads to a dramatic decrease in the total number of undifferentiated spermatogonia, with a lesser decrease in SSC numbers (Parker et al. 2014).

Acting downstream from GDNF in the spermatogonial lineage are kit ligand and its receptor, c-kit. Experiments with mutant c-kit protein showed that c-kit is necessary for the irreversible transition from type A undifferentiated spermatogonia to differentiating type A1 (de Rooji et al. 1999). c-kit is also critical for the development of type A differentiating spermatogonia from A1 to A4. In vivo experiments administering anti- c-kit antibodies caused depletion of A1 to A4 spermatogonia while the undifferentiated spermatogonia remained intact (Yoshinaga, 1991). Thus, c-kit is vital for and a marker of spermatogonial differentiation. Parker et al. showed that with the inhibition of GDNF for 7 days, the fraction of A_s undifferentiated GFRα1$^+$ cells expressing c-kit increased from 0.008 to 0.40. Our lab noted that control mice have few cells in which c-kit is co-localized with the undifferentiated spermatogonia cell surface marker GFRα1. In theory cells expressing c-kit are type A differentiating spermatogonia, while cells expressing GFRα1 are type A undifferentiated spermatogonia, and cell expressing both c-kit and GFRα1 are undifferentiated spermatogonia undergoing the transition from $A_{s/pr/al}$ to A1.

The question posed by the Wright lab is how do GFRα1$^+$ spermatogonia respond to a reintroduction of GDNF signaling after 9 days of inhibition. To address this, we used an *in vivo* chemical-genetic approach inhibiting GDNF signaling and investigating the role it plays on SSCs by measuring the cell number, differentiation, and replication of undifferentiated spermatogonia over time. GFRα1 functions at the cell surface as the ligand-binding subunit of the GDNF receptor, and signal transduction then occurs through the receptor tyrosine kinase RET. RET signaling requires an activating phosphorylation in its active site (see Figure 3). In our mutant mouse model the RET

active site possess a mutation which under normal conditions has no effect on the animal's spermatogonia. But pharmacological intervention with a bulky-ATP inhibitor blocks the RET active site from activating phosphorylation effectively inhibiting GDNF signaling (Figure 3.)(Savitt et al., 2012). Inhibiting GDNF drastically perturbs the niche and therefore the SSCs. Our work utilizes this perturbation to study the response undifferentiated spermatogonia measuring cell number, replication, and specifically in this thesis, differentiation. GDNF is known to maintain undifferentiated spermatogonia *in vitro* and *in vivo*, the thesis analyzes the differentiation of these undifferentiated cells after inhibition and restoration of GDNF signaling.

Figure 3.

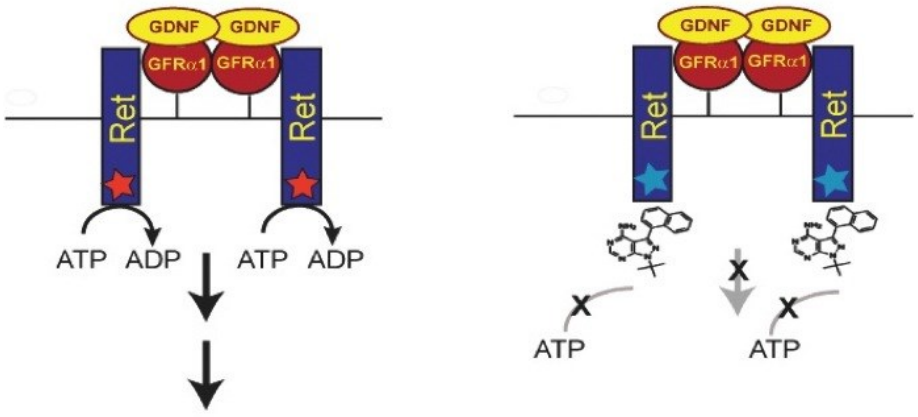


Figure 3. This is an illustration of the interacting of GDNF-GFRα1-Ret at the cell surface. The left illustration demonstrates activating phosphorylation and signaling under normal conditions. The right illustration demonstrates the drug bulky ATP-inhibitor.

MATERIALS AND METHODS

-Animals and Their Treatment

The murine model used were black6 mice with a V805A mutation in the ATP binding site of the tyrosine kinase Ret (Savitt et al., 2012). Homozygous Ret mutant mice were identified used polymerase chain reaction analysis of their genomic DNA. The primers used crossed the 5' LoxP site of the targets construct:

Ret F (36580): CCTTGGGCCTGCTGAGCACGGG

RET R (36858): GGAGGCAGGAAGGCCTGTGC

PCR conditions were as follows; 4 minutes at 95 C followed by 35 cycles of 30 seconds at 95 C, 45 seconds at 57 C, 45 seconds at 72 C, followed by a 7-minute incubation at 72 C.

To inhibit GDNF signaling Ret mutant (V805A) aged 90-120 days were injected subscapularly once daily for nine day at 24 hour intervals with 43.7 mg/kg body weight 1NA-PP1-HCl, which is dissolved in ethanol at a concentration of 62 mg per ml (described at NA-PP1 henceforth) which was synthesized and prepared for injection solution as described in (Savitt et al., 2012). Control mice were injected with 100 microliters of vehicle plus ethanol (saline:cremophor:ethanol::28:8:3.6) per 10-grams of body weight.

Following treatment 4 to 7 animals were sacrificed at Day 10 (one day following the last injection of NA-PP1), Day 14, and Day 22. Results were gathered from two separate trials over the course of 2015-2016. The Johns Hopkins University Institutional Animal Care and Use Committee approved this protocol.

-Collection of tissue and Immunocytochemistry

Whole testes were dissected from the animal, weighed, and 12-20 seminiferous tubules per animal were fixed for 1 hour at 4 C in 10% paraformaldehyde 10% methanol in phosphate-buffered saline (PBS; pH=7.3-7.4 pH). Methanol was used to perfuse the cell membrane exposing the intracellular c-Kit epitope for antibody binding. Specimens were fixed within 30 minutes of sacrifice to mitigate degradation of relevant proteins.

After fixation the tubules were washed 6 times for 15 minutes each in PBS at room temperature. Immediately afterwards, the samples were blocked for 1 hour at room temperature in 1% Bovine Serum Albumin (BSA) in PBS. The samples were then incubated in primary antibodies overnight at 4 C. c-kit rat anti-mouse (CD117, CBL1360, clone ACK2) at a 1:50 dilution in 250 microliter of PBS-BSA 1%- Triton x-100 at 0.1% (PBS-BT) per well. GFRα1 goat anti-mouse (AF560, R & D systems) at 1:100 dilutions in 250 microliter PBS-BT per well. The GFRα1 primary antibody was a polyclonal IgG that had been affinity purified against GFRα1, and the c-Kit was a rat monoclonal IgG2b, kappa isotype.

The next day the samples were washed 6 times for 15 min each wash in PBS-BT at room temperature. After that secondary antibodies were applied for two hours at room temperature void of light. The secondary antibodies were made in 1:200 dilutions in 250 microliters of PBS-BT. c-Kit secondary is an AlexaFluor 488-conjugated donkey anti-rat IgG, and GFRα1 is an AlexaFluor 555 conjugated donkey anti-goat IgG. The tubules were again washed 6 times for 15 min each in PBS-BT at room temperature. Finally 5-10

tubules per animal were then whole-mounted on glass slides with VectaShield fluorescence.

c-Kit and GFRα1 were selected because they are markers for differentiating and undifferentiated type A spermatogonia respectively. In normal, mature testes, c-Kit expression is present from A1 to preleptotone spermatocytes, and is necessary for undifferentiated single, paired, aligned spermatogonia transitioning to differentiating type A1 spermatogonia. GFRα1 is a marker of undifferentiated spermatogonia and is expressed in A_s, A_{pr}, and A_{al}.

-Microscopy

The immunofluorescence staining of seminiferous tubules was imaged using a Zeiss LSM 710NLO-Meta Confocal Microscope (25X lens). Cells in the basal aspect of seminiferous tubules were imaged according to the confocal specifications shown below. Signals produced by the green and red fluorchromes were captured separately, and subsequently merged.

2-10 tubules were scanned and imaged per animal, dependent on length and number of GFRα1$^+$ cells present (i.e. controls had greater than 50 GFRα1$^+$ cells per tubules, day 10 on average less than 10 cells per tubule).

c-Kit Staining: 488 (Green Laser):

- Laser: 75.0%
- Pinhole: 46.5 units
- 1.00 Airy Units=2.2 micron section
- Gain Master: 750
- Digital Master: 125
- Digital Offset: 1.0

GFR α1 Staining 543 (Red Laser):

- Laser: 15.0%
- Pinhole: 42.9 units
- 0.81 Airy Units=2.2 micron section
- Gain Master: 760
- Digital Master: 125
- Digital Gain: 1.0

-Statistics

Data from the two replicate experiments were pooled and data analyzed both by ANOVA and a nested ANOVA using StatView. In this analysis, day and cell type were nested within the effects of treatment. Differences between means were tested by Fisher's PLSD test.

RESULTS

-Confocal Microscopic Identification of Cells expressing GFRα1, c-kit or both marker proteins in control seminiferous tubules and in tubules collected at day 10 (1 day after the last injection of 1Na-PP1), or at days 14 or 22.

We used confocal microscopy to image between 2 and 10 seminiferous tubules per animal in order to gain a clear understanding of the relative numbers of GFRα1^{+}, c-kit^{-}, GFRα1^{+}, c-kit^{+} and GFRα1^{-}, c-kit^{+} cells, the relative amount GFRα1 expressed per cell, and the morphologies of the cells in the different experimental groups. The four groups control, day 10, day 14, and day 22 showed apparent differences qualitatively and quantitatively in the number, morphology, and staining intensity of GFRα1^{+} cells. For the purposes of illustration, we present the results for GFRα1, c-kit separately and also merged in one image.

Image 1. Control Mice

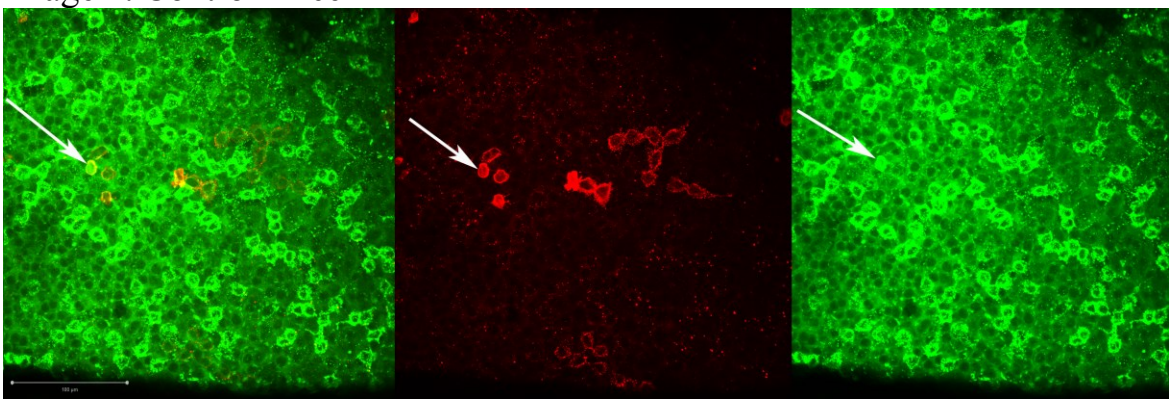


Image 1. Control Mice. Left, combined red/green image. Middle, red GFRα1^{+}. Right, green c-Kit^{+}. The white arrow indicates the only cell in this image expressing both. Approximately 2-4 tubules per animal were analyzed per the control group.

Image 1 is a representative image of a tubule from a control animal. 9 control animals were injected with vehicle on the same schedule as treatment animals, and collected and imaged using the same methods. The left most image is a composite of both green (c-kit) and red (GFRα1) channels. The center image is strictly the red GFRα1 channel, and the right is strictly the green c-kit channel. Note that most of the GFR□1^{+} cells do not express c-kit. However, the white arrow identifies a GFRα1^{+} cell that also expressed c-kit.

In the center of the images there are two separate chains of A_{al} cells in the upper and lower middle segment of seminiferous tubule. Aligned chains of GFRα1^{+} cells are seen throughout the control tubules. Chain lengths of four and eight intercellular-bridged cells are the most common, although longer chains are regularly observed. The grouping of cells to the left of the images is a fragmenting chain that contains one c-kit^{+} GFRα1^{+} co-stained cell. Co-stained cells normally occur in or near chains of cells, and often only one marker (c-kit or GFR□1) is intensely fluorescing. The left panel shows that co-stained cells are rare in control, while long chains of brightly green stained c-kit^{+},

GFR□1^{-} spermatogonia are numerous in controls. Looking at the right panel, c-kit^{+} cells are ubiquitous in control tubules existing in chains (Image 1) of cells connected by intercellular bridges morphologically similar to chains of GFR□1^{+} cells.

Image 2. Day 10 Mice

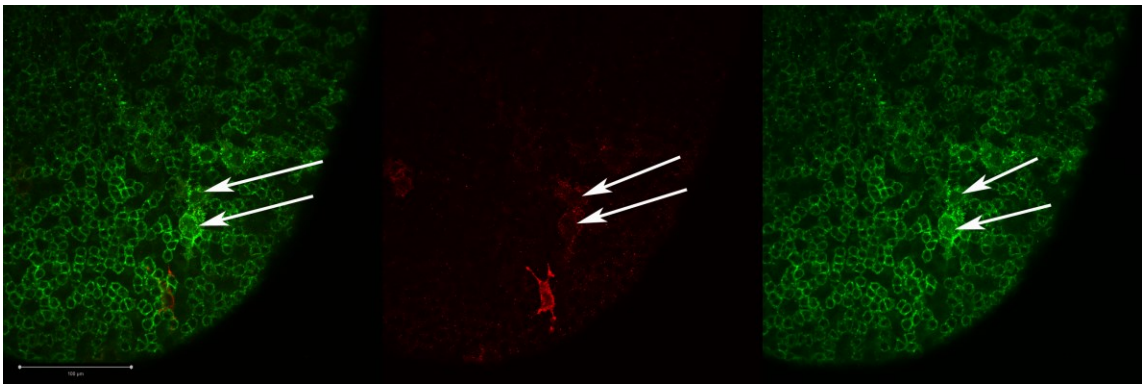


Image 2. Day 10 mice. Left, combined red/green image. Middle, red GFRα1^{+}. Right, green c-Kit^{+}. The white arrows indicate two cells in this image expressing both markers. The less intense c-kit staining at the top of the image is due to mounting of the tubule section on the slide and can be observed in greater intensity when the microscope is focused on that section.

This is a representative image from a Day 10 animal, and thus, sacrificed 24 hours after the last of 9 daily injections of 1NA-PP1. Day 10 has notably fewer GFRα1^{+} cells present than any other time point because of this approximately 6 to 10 tubules were analyzed per animal. Almost all of the GFRα1^{+} cells at this time point fluoresce noticeably less brightly than control cells, and chains longer than four cells are very rare. Like the cells in Image 2, the morphology of day 10 GFRα1^{+} cells is generally larger and more asymmetrical than the circular A_{al} control chains. Of the remaining GFRα1^{+} cells at day 10 co-staining with c-kit is more numerous than any other time point. The white arrows indicate two co-stained cells that are faintly expressing GFRα1. Most of the GFRα1^{+} c-kit^{+} cells at this time point are faintly expressing GFRα1 and strongly

expressing c-kit. Generally, c-kit staining is present throughout the tubules is similar to control with chains or tiling (seen here in Image 2) of cells.

Image 3. Day 14 Mice

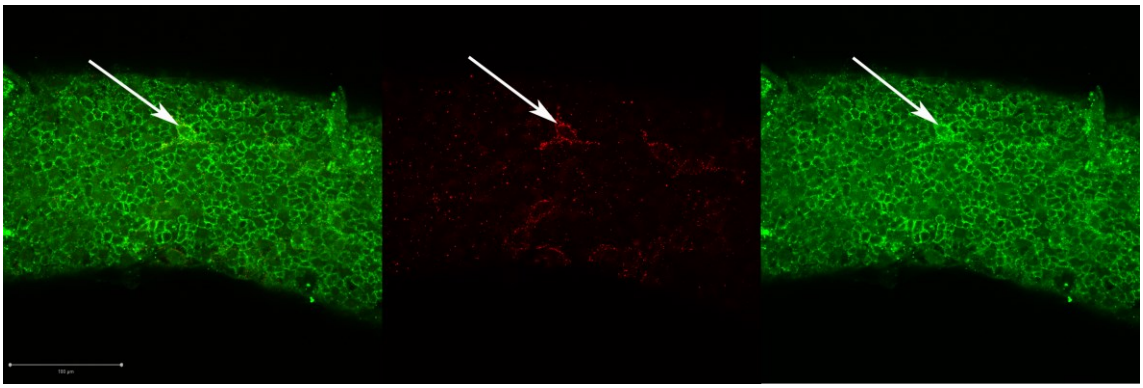


Image 3. Day 14 Mice. Left, combined red/green image. Middle, red GFRα1^{+}. Right, green c-Kit^{+}. The white arrow indicates the only cell in this section expressing both. This tubule is smaller than the others represented in this thesis it is a 25x magnification image.

This is a representative image from a Day 14 animal, and, thus, sacrificed 5 days after the last of 9 daily injections of 1NA-PP1. At day 14 GFRα1^{+} cells are more numerous than the sparsely populated day 10 tubules but still drastically lower in number than in control tubules. It is common for GFRα1^{+} cells at day 14 to fluoresce noticeable less brightly than cells in control mice. In this image faint chains of cells are present at the bottom-middle and right of the image. Short chains like these of 4-8 cells are common but longer chains longer than 8 are very rare. The c-kit staining throughout the tubules is similar to control with chains or tiling of cells ubiquitous throughout the tubules. Co-staining at day 14 is similar to day 10 with co-stained cells showing faint GFRα1 fluorescence and brighter c-kit signal.

Image 4. Day 22 Mice

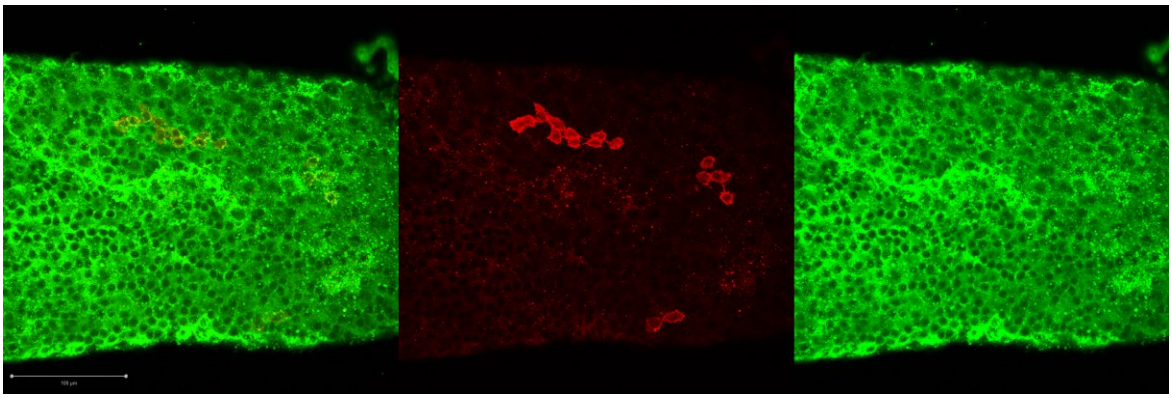


Image 4. Day 22 Mice. Left, combined red/green image. Middle, red GFRα1^{+}. Right, green c-Kit^{+}.

This is a representative image from a day 22 animal, and, thus, sacrificed 13 days after the last of the daily injections of 1NA-PP1. GFRα1^{+} are frequently in chains or clumps of over 16 cells, which are not often seen in controls. The intensity of fluorescence of GFRα1^{+} cells is similar to control cells, unlike many of the day 10 and 14 cells. Interestingly, c-kit cells are greatly reduced in number in foci of the tubules, but do appear in normal chains in other parts of the tubule. The reduction in c-kit^{+} cells is illustrated in this panel. At day 22 entire 4-8 cell chains are GFRα1^{+} c-kit^{+}, unlike control where only a cell or two associated with a chain is GFRα1^{+} c-kit^{+}.

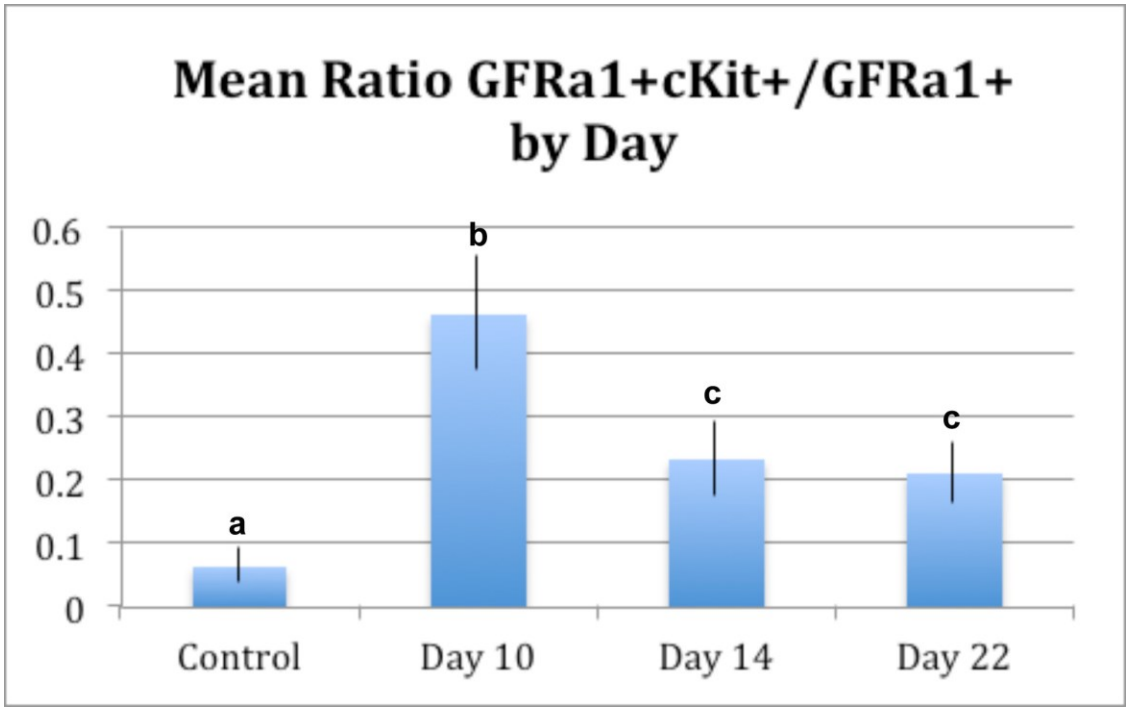
-Quantitative Analysis of the Changes in Differentiation of GFRα1^{+} spermatogonia following restoration of GDNF signaling.

The analysis described suggested that following restoration of GDNF signaling, the fraction of GFRα1^{+} spermatogonia that were differentiating was reduced. To test this quantitatively, we enumerated both GFRα1^{+} c-kit^{-} spermatogonia and GFRα1^{+} c-kit^{+} spermatogonia in order to calculate the fraction of all GFRα1 expressing c-kit (i.e.

differentiating) in control mice, and in mice collected on days 10, 14 and 22 of the experiment.

Graph 1 illustrates general trends in the mean ratio of c-kit$^+$ GFRα1$^+$ spermatogonia as a fraction of total GFRα1$^+$ spermatogonia for controls and for each treatment group. The animals collected at day 10 exhibited the highest mean ratio of c-Kit$^+$ GFRç1$^+$/GFRα1$^+$ spermatogonia, with a 7.25-fold increase over control. This is indicative of the effect losing GDNF signaling has on undifferentiated spermatogonia as the last treatment injection was only 24- hours prior. Over time the ratio decreases to 3.6-fold and 3.3- fold above control at Day 14 and Day 22 respectively. Scheffe's tests confirmed a significant difference between control and Day 10 of treatment, with a p value <0.0001 (null defined at p value <0.05) (See Appendix table 1.). Fischer's PLSD also confirmed a significant difference between control and Day 14 with p value 0.0056. (see APPENDIX for further statistical analysis)

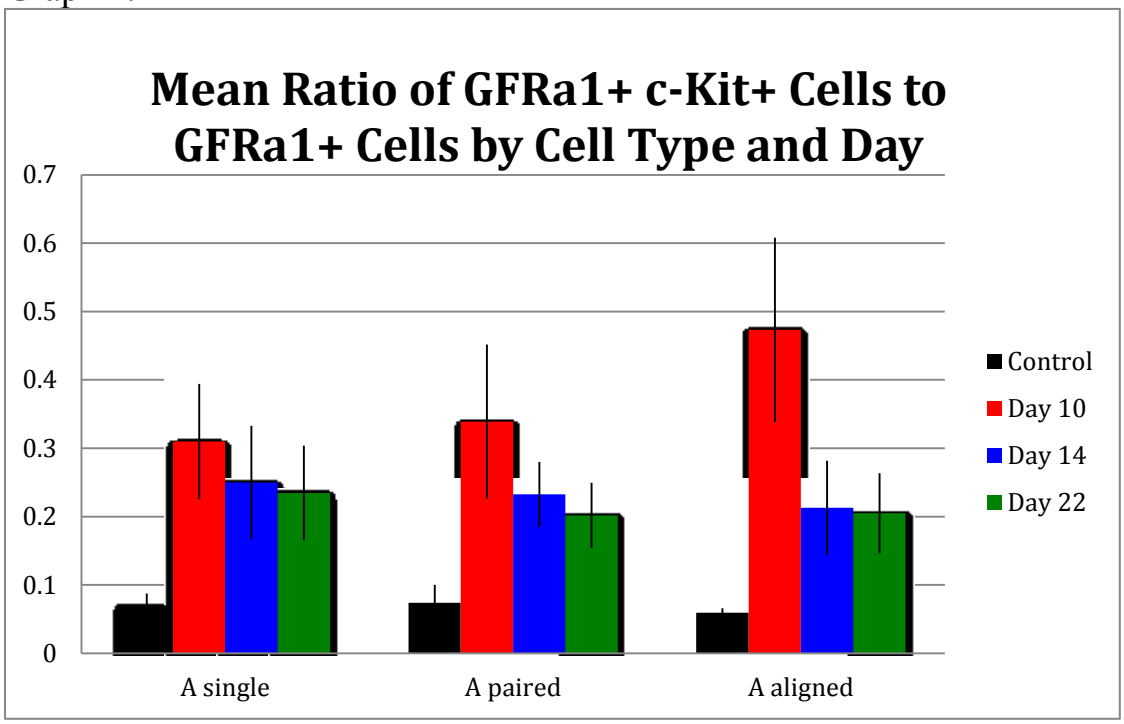
Graph 1.



Graph 1. The mean ratio of is reported by assessing the ratio of GFRα1+cKit+ /GFRα1+ for each animal and averaging the ratios together by treatment group. Day 10; 7.25-fold increase in the ratio of spermatgonia marked for differentiation compared to control. Day 14;3.6-fold increase. Day 22; 3.3-fold increase. Bars with different superscripts differ statistically.

Graph 2 stratifies each treatment group by cell type A_s, A_{pr}, A_{al} and there are interesting differences between cell types during restoration of GDNF signaling. In A_s, the population classically hypothesized to contain most of the self-renewing stem cells, there is minimal change in the fraction of undifferentiated spermatogonia between days 10 and 22, with ratios of 0.30 and 0.24 respectively. Meanwhile the A_{al} population, considered to contain mostly progenitor type cells, has a more drastic response to GDNF signaling restoration by Days 14 and 22. The mean ratio of c-kit$^+$ GFRα1$^+$ / GFRα1$^+$ spermatogonia drops from 0.47 at day 10, to 0.21 at day 14, and 0.20 by day 22. A_{pr} display a response immediate to the A_s and A_{al} cell types when GDNF signaling is restored.

Graph 2.



Graph 2. Fraction of GFRα1+cKit+ spermatogonia out of total GFRα1+ spermatogonia at control and three time points post treatment by cell type reported as the mean ratio. Day 10 (red bars) of treatment is associated with an increase in the ratio of cells moving toward differentiation for all types of spermatogonia 4.4-fold for As and 8-fold for A_{al}. By day 22 progenitor A_{al} spermatogonia show a dramatic decrease in the ratio of GFRα1+cKit+ differentiating spermatogonia to 3.6-fold above control. While the A_s population is more resistant to change remaining nearby at 3.4-fold above control. Nested ANOVA showed that there was overall a significant difference between the different treatment groups.

DISCUSSION

The research described in this thesis was motivated by the hypothesis that restoring GDNF signaling after 9 days of its inhibition would cause the remaining undifferentiated spermatogonia to suppress differentiation thereby restoring numbers of SSCs and progenitor spermatogonia in the testis. The work is part of the Wright lab's larger goal to elucidate the role GDNF plays in rebuilding a depleted SSC population, and investigate possible causes and treatments of a type of male infertility called Stertoli Cell-Only. This phase of research involved inhibiting and restoring GDNF signaling and measuring the response in cell number, replication, and differentiation over time. The data generated in this thesis show that after restoration of GDNF signaling, differentiation

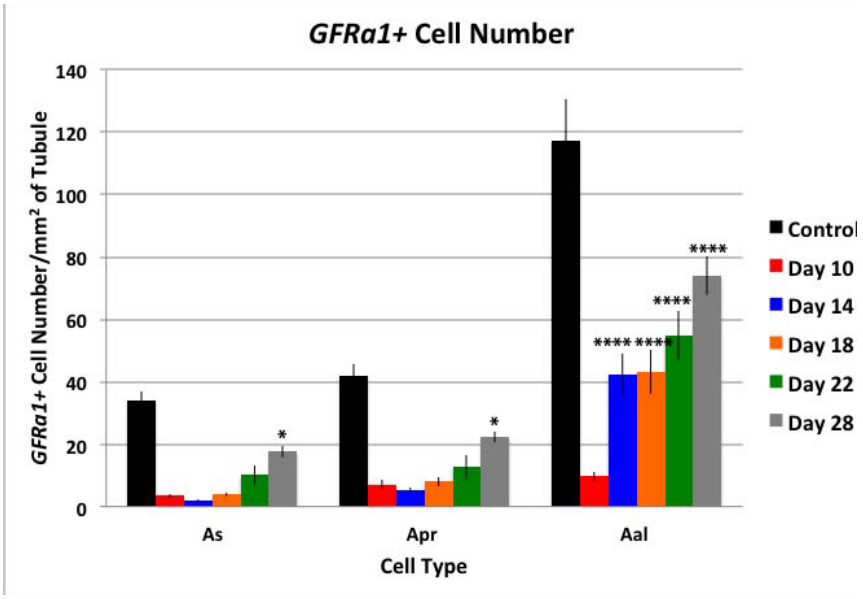
of GFRα□ □spermatogonia, as measured by co-expression of c-kit, is suppressed. However, even 13 days after restoration of signaling, the fraction of all GFRα□ □spermatogonia that are also c-kit$^+$ is still greater than controls. This is most evident in the A_s population. The significance of these results are best understood in the context of the data of Nicole Parker, a Ph.D. student in the Wright laboratory. In similar experiments she measured cell number and replication of GFRα1$^+$ spermatogonia, and the combined results of the lab implicate how SSC repopulate the testis.

The results of this thesis show a demarcation between A_s and A_{al} populations' differentiation during restoration of GDNF signaling. The loss of GDNF signaling leads to a large decrease in the number of GFRα1$^+$ cells. Many of the remaining GFRα1$^+$ exhibited less intense fluorescence, and of those cells a large fraction exhibited c-kit co-staining. GDNF is known *in vitro* and *in vivo* to support undifferentiated spermatogonia. Our data show that after 9 days of inhibited GDNF signaling, the fraction of GFRα1$^+$ spermatogonia that express c-kit is increased 5.5-fold, indicating that differentiation is one cause for the loss of the cells. However, on day 22, this fraction is reduced to 3.3-fold. Quantitatively, the GFRα1$^+$ A_{al} population has a drastic drop in expression in c-kit as signaling from 0.47 at day 10 to 0.20 at day 22. While in the A_s population only drops from 0.30 to 0.24 over the same time period, a somewhat perplexing result since SSC are considered to be a subset of this population. None-the-less, our results demonstrate that restoration of GDNF signaling gradually suppresses the increased rate of differentiation of A_s, $A_{pr,}$ and A_{al} spermatogonia.

The importance of these observations is evident when they are viewed in the context of data generated by Nicole Parker, a Ph.D. student in the Wright laboratory, who

used the same chemical-genetic approach mentioned in this thesis to inhibit GDNF signaling for nine days and study the subsequent changes in numbers of GFRα1$^+$ spermatogonia and in their replication. In fact many of the samples were collected from the same animals studied here.

Long Term Inhibition of GDNF signaling decreases GFRα1 cell number and the cell number increases with recovery of signaling

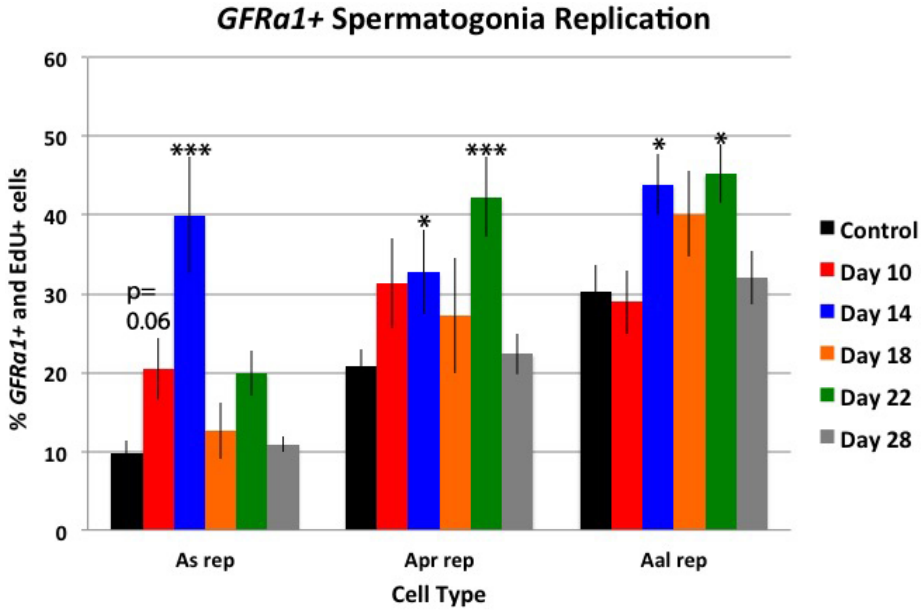


Graph 3. The graph indicates the mean number of GFRα1$^+$ spermatogonia per mm^2 of the seminiferous tubule and the standard error of the mean. For each cell type at recovery time points the cell number was significantly reduced from controls indicated by 2-way ANOVA. A significant increase in recovered cell number was shown at day 28 in A_s and A_{pr} cells compared to day 14 and at all days A_{al} compared to day 10. (*) $p<0.05$ (****) $p<0.0001$ by post-hoc comparison tests.

Nicole first asked how the A_s, A_{pr}, and A_{al} GFRα1$^+$ spermatogonia responded by cell number. She used very similar immunocytochemistry and microscopy techniques to the ones described in this thesis. Nicole showed that inhibition of GDNF signaling for nine days drastically reduces the number of the GFRα1$^+$ in all cell. The populations of A_s and A_{al} GFRα1$^+$ spermatogonia seem to responded independently to inhibition and

restoration of GDNF signaling. The A_{al} population sees a statistically significant increase in population at day 14 and continues a steady increase in number thereafter. While the A_s population does not exhibit a statistically significant increase until day 28.

Replication of GFRα1+ spermatogonia changes in the recovery of GDNF signaling



Graph 4. This graph shows the effect of a nine day treatment of NA-PP1, inhibition of GDNF signaling, on replication of GFRα1$^+$spermatogonia during the observed recovery. The mean and standard error of mean in replication is displayed. The data shows a significant increase in replication from control indicated by 2-way ANOVA. (*) $p<0.05$, (***) $p<0.001$.

Nicole also examined replication in the same group of animals using EdU staining to identify replicating cells. All populations of GFRα1$^+$ spermatogonia measured here respond to the restoration of GDNF signaling with an increase in replication. Interestingly the A_s population has the most drastic response at day 14. The A_s population has a large 4- fold increase in replication at day 14 of the GDNF recovery experiment then drops near control levels by day 18. While A_{pr} and A_{al} populations see only a 1.5-

fold increase in replication at day 14 and maintain similarly high replication levels through day 22.

Form Nicole's data and results of this thesis a hypothesis can be formed, the undifferentiated spermatogonia response to a restoration of GDNF signaling by building progenitor populations first. Her data shows the A_s population, classically thought to contain the SSCs, reacts to restoration of GDNF signaling by increasing replication drastically at day 14. However, cell numbers are not significantly increased until day 28. In contrast the A_{al} population, classically considered undifferentiated progenitor transit amplifying spermatogonia, responses to a reintroduction of GDNF signaling with a slight increase in replication and a quick dramatic increase in cell number. Combining Nicole work with the results described in this thesis implies a restoration of GDNF signaling has different affects on SSC and undifferentiated transit amplifying progenitor spermatogonia. The A_s population acts by increasing replication and differentiation, producing cells that leave the population functioning for transit amplification. The A_{al} population responses to re-introduction of GDNF signaling by suppressing differentiation to increase cell number. Hypothetically, this re-establishes a large pool of transit amplifying spermatogonia in an effort to continue spermatogenesis. However, the combined work demonstrates that after the restoration of the progenitor spermatogonia, the numbers of A_s cells increases, and presumably, the SSCs. The combined work implies a restoration of GDNF signaling has different effects on SSC and undifferentiated transit amplifying progenitor spermatogonia. This nuanced response indicates GDNF may act in coordination with other extrinsic signaling molecules in order to sequentially rebuild the numbers of transit amplifying progenitor spermatogonia and then the pool of SSCs.

APPENDIX

Fisher's PLSD for Column 4
Effect: Day
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
Control, Day 10	-.306	.117	<.0001	S
Control, Day 14	-.164	.115	.0056	S
Control, Day 22	-.146	.120	.0181	S
Day 10, Day 14	.142	.112	.0129	S
Day 10, Day 22	.160	.117	.0079	S
Day 14, Day 22	.018	.115	.7560	

Fisher's PLSD for Column 4
Effect: Cell type
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value
Single, Paired	.004	.100	.9337
Single, Aligned	-.022	.100	.6634
Paired, Aligned	-.026	.100	.6043

Scheffe for Column 4
Effect: Day
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
Control, Day 10	-.306	.168	<.0001	S
Control, Day 14	-.164	.165	.0514	
Control, Day 22	-.146	.173	.1300	
Day 10, Day 14	.142	.160	.1002	
Day 10, Day 22	.160	.168	.0677	
Day 14, Day 22	.018	.165	.9921	

References

Brinster RL and Avarbock MR. 1994. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91(24):11303-7.

Brinster RL and Zimmermann JW. 1994. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91(24):11298-302.

Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S. 2005. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73(5):1011-6.

de Rooij DG. 2001. Proliferation and differentiation of spermatogonial stem cells. Reproduction 121(3):347-54.

de Rooij DG and Griswold MD. 2012. Questions about spermatogonia posed and answered since 2000. J Androl 33(6):1085-95.

de Rooij DG and Russell LD. 2000. All you wanted to know about spermatogonia but were afraid to ask. J Androl 21(6):776-98.

de Rooij DG, Okabe M, Nishimune Y. 1999. Arrest of spermatogonial differentiation in jsd/jsd, Sl17H/Sl17H, and cryptorchid mice. Biol Reprod 61(3):842-7.

DEL CASTILLO EB, TRABUCCO A, DE la BALZE FA. 1947. Syndrome produced by absence of the germinal epithelium without impairment of the sertoli or leydig cells. J Clin Endocrinol Metab 7(7):493-502.

Griswold MD and Oatley JM. 2013. Concise review: Defining characteristics of mammalian spermatogenic stem cells. Stem Cells 31(1):8-11.

Gul U, Turunc T, Haydardedeoglu B, Yaycioglu O, Kuzgunbay B, Ozkardes H. 2013. Sperm retrieval and live birth rates in presumed sertoli-cell-only syndrome in testis biopsy: A single centre experience. Andrology 1(1):47-51.

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T. 2005. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72(4):985-91.

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fassler R, Shinohara T. 2008. Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3(5):533-42.

Nagano MC. 2003. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69(2):701-7.

Oakberg EF. 1971. Spermatogonial stem-cell renewal in the mouse. Anat Rec 169(3):515-31.

Oatley JM and Brinster RL. 2012. The germline stem cell niche unit in mammalian testes. Physiol Rev 92(2):577-95.

Oatley JM and Brinster RL. 2008. Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263-86.

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL. 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136(7):1191-9.

Parker N, Falk H, Singh D, Fidaleo A, Smith B, Lopez MS, Shokat KM, Wright WW. 2014. Responses to glial cell line-derived neurotrophic factor change in mice as spermatogonial stem cells form progenitor spermatogonia which replicate and give rise to more differentiated progeny. Biol Reprod 91(4):92.

Savitt J, Singh D, Zhang C, Chen LC, Folmer J, Shokat KM, Wright WW. 2012. The in vivo response of stem and other undifferentiated spermatogonia to the reversible inhibition of glial cell line-derived neurotrophic factor signaling in the adult. Stem Cells 30(4):732-40.

Savitt J, Singh D, Zhang C, Chen LC, Folmer J, Shokat KM, Wright WW. 2012. The in vivo response of stem and other undifferentiated spermatogonia to the reversible inhibition of glial cell line-derived neurotrophic factor signaling in the adult. Stem Cells 30(4):732-40.

Shinohara T, Avarbock MR, Brinster RL. 1999. Beta1- and Alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96(10):5504-9.

Silber SJ, Johnson L, Verheyen G, Van Steirteghem A. 2000. Round spermatid injection. Fertil Steril 73(5):897-900.

Takizawa H and Manz MG. 2012. In vivo divisional tracking of hematopoietic stem cells. Ann N Y Acad Sci 1266:40-6.

Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T, Nishikawa S. 1991. Role of c-kit in mouse spermatogenesis: Identification of spermatogonia as a specific site of c-kit expression and function. Development 113(2):689-99.

Scholarly Life

-Hughesville, Pennsylvania

I was born July 15, 1992 in central Pennsylvania. From childhood I had strong interest in the sciences and outdoor life. My biology, geology, geography, and history strongly overlapped with my love of the outdoors. Later in high school I further explored my love for science while excelling in athletics.

-Baltimore, Maryland

As an undergrad at Johns Hopkins University I majored in Public Health Studies and was an active member of the JHU football team. My bachelor's degree brought me to the Bloomberg School of Public Health. Specifically, the department of Biochemistry and Molecular Biology (BMB), where I found deeper study of biology re-sparked my adolescent interest. Soon after beginning the ScM program in the BMB department I started this research in the lab of William Wright, PhD. I was intrigued by his work because it fell into the fields of reproductive biology and stem cell biology. Along with classwork and research in the department of BMB, I also contributed as teaching assistant for the classes "Pandemic Influenza" and "Cellular and Molecular Mechanism of Reproduction."